The handle http://hdl.handle.net/1887/65601 holds various files of this Leiden University dissertation.

**Author:** Veen, T.P.M. van  
**Title:** GDE transmembrane ecto-enzymes : novel signaling functions through GPI-anchor cleavage  
**Issue Date:** 2018-09-19
Chapter 6

Summary and Discussion
Discussion

The GPI anchor, an enzyme substrate or a membrane attachment signal?

In general, the membrane has a fluid-like nature in which different proteins and lipids interact to influence its organization, dynamics and function. In this fluid-like environment, rafts were postulated to serve as stable sorting platform for specific lipids, GPI-anchored proteins and other proteins, that “float” on top of the membrane [271, 272]. Although demonstrated in artificial membranes, evidence for native raft domains was never convincingly shown, which raises the question why proteins are GPI-anchored [268, 273-276]. A vast amount of studies, using a wide variety of techniques (i.e. fluorescence recovery after bleaching (FRAP), Fluorescence energy transfer (FRET), super resolution imaging or single particle analysis), have focused on the contribution of the GPI-anchor to specific localization in transient micro-domains. To date a picture has emerged in which GPI-anchored proteins have diffusion coefficients comparable to membrane lipids, yet, only a part of the total fraction is mobile and diffuses freely, albeit in constricted domains [46, 268, 277-281]. In addition, recent studies suggest that (indirect) interaction of GPI anchors with the cortical actin contributes to the formation of domains [194, 282-285]. Although still under debate, these results indicate that some order or microdomains, where GPI-anchored proteins reside, may be present.

Remarkably, localization to microdomains alone does not comply with the complexity of the GPI-anchor, as the contribution of the inositol and mannose groups in the GPI anchor remain unanswered or even ignored. Hence, bacterial phospholipases and bacterial toxins, that specifically recognize this part of the GPI anchor, are of special interest and raise the question whether the primary function of the structure is to be a substrate for mammalian enzymes [286, 287]. Chapter 2 and 3 endorse the latter and identify GDE2 and GDE3 (unlike other GDE family members) as trans-membrane GPI-hydrolyzing phosphodiesterase. An additional observation that favors the hypothesis that GPI anchors serve as a ligand, is the finding of various circulating, but still active GPI-anchored proteins (partially described in Addendum Chapter 3) [189, 222, 288]. In line with the previous, GPI-anchored proteins that are resistant to a PLC attack but still harbor a lipid tail, can have a deleterious effect during fertilization [9, 33].

Various enzymes can release GPI-anchored proteins including: GDE2 and GDE3 [17, 110, 116], a recently identified phospholipase A2 [77], the more enigmatic GPI-PLD, Notum and ACE [16, 18, 65] and various metalloproteinases [289, 290]. To distinguish their relative role, in releasing GPI-anchored proteins, more research is needed. For example; to sperate GPI-anchored proteins released by metalloproteinases or phospholipases (-C and -D), the presence
Summary and Discussion

or absence of the glycan core should be carefully evaluated (e.g. by HPLC or alpha-toxins). Previous studies on circulating GPI-anchored proteins have often used metalloproteinase inhibitors to proof the underlying molecular mechanism but these inhibitors rely on metal chelation which is a critical component in GPI anchor synthesis as well as the cleavage by phospholipases [65, 291, 292]. Therefore, the contribution of phospholipases in releasing GPI-anchored proteins could be underestimated and caution should be taken when evaluating these results. In addition, GPI-anchored proteins released by phospholipases can still undergo cleavage by metalloproteinases, potentially masking GDE activity.

**GPI-specific phospholipases C or D?**

Ever since the discovery of bacterial GPI-PLC, mammalian GPI specific phospholipases (PLC or PLD) have been proposed to serve an important function in the regulation of GPI-APs; reducing cell-surface expression while at the same time generating a soluble protein. A notion supported by the discovery of various soluble GPI-APs in body fluids [293], tissues [294, 295], urine [296], and cerebrospinal fluid [263, 297]. Evidence for the existence of GPI-APs released by GPI-PLCs has been obtained using an anti-CRD antibody that selectively recognizes the epitope of a GPI-PLC cleaved protein [295]. Using this antibody, multiple soluble GPI-APs were detected which proofs the existence of an unidentified mammalian GPI-PLC [295, 296, 298, 299]. In Chapter 2 we performed a mass-spec analysis on the released product of GDE3 and found that GDE3 cleaves in a phospholipase C dependent manner, thus identifying GDE3 as the first mammalian GPI-PLC. Interestingly however, immunoblot analysis of GPI-anchored RECK released by GDE2, with an anti-CRD antibody proofed that GDE2 is not a classical type C-phospholipase [110]. In line with the previous, GDE3 attacks glycerophosphoinositol as a phospholipase C while cleavage of glycerophosphocholine by GDE2 occurs in a phospholipase D like manner [99, 100]. Together these observations hint to divergent kinetics between the evolutionary conserved GDE2 and GDE3. The latter is of special interest since it could account for GDE2 and GDE3 substrate specificity; PLC’s, unlike PLD’s, are sterically hindered by structural heterogeneity of the GPI moiety and therefore cleave only a selective pool of GPI-anchors [265, 300]. One such GPI modification, palmitoylation of the inositol group, occurs during biosynthesis in the ER and is later removed in the Golgi by PGAP1 [32, 33]. Interestingly, mice lacking PGAP1 have severe defects including infertility due to an accumulation of GPI anchors on the membrane. This suggests a role for an, as yet unidentified, GPI-PLC in releasing GPI-anchors [9, 33]. Further studies using PGAP1 mutant cell lines could answer the question whether GDE2 is indeed a GPI-PLD (i.e. Is GPI-anchored cleavage by GDE2 attenuated in PGAP1 mutant cell lines?). It is of note that in our hands, GDE2 cleaves only a selection of GPI anchors compared to GDE3. Hence, factors other than enzyme kinetics are likely to affect cleavage.
Until now GDE studies have focused on the release of GPI-anchors and not on the remaining lipid in the plasma membrane. PLC’s or PLD’s generate a specific lipid when cleaving a GPI-anchored protein; diacylglycerol (DAG) or phosphatidic acid (PA) respectively. DAG is of special interest because it is a second messenger signaling lipid that activates protein kinase C (PKC), which consequently leads to phosphorylation of downstream substrates. Interestingly, recent research has shown that a pool of DAG is present in the plasma membrane outer leaflet and fast trans bilayer movement could occur [301]. GDE-mediated protein release could therefore directly affect intracellular DAG signaling. More research is needed to assess if DAG generated by GDE3 in the outer leaflet has a signaling function on its own.

The role of the GDE signaling axis in cancer

Selective GPI-anchored proteins as well as total circulating GPI-anchored protein levels have been described as plasma biomarkers for specific cancers (e.g. mesothelin and carcinoembryonic antigen) [287, 302]. Also, expression of multiple enzymes involved in GPI-anchor synthesis (GPAA1, PIGT and PIGU described in the introduction) is increased in different cancer types and is associated with increased cell division [303-305]. Interestingly, in breast carcinomas, increased expression of these enzymes was found to increase overall GPI-anchored protein expression, thereby connecting GPI synthesis and expression in cancer cells [306]. Based on these results, elevated circulating GPI-anchored proteins can reflect an increase in cleavage or simply increased synthesis of the GPI anchor itself. Research on ovarian cancer derived, circulating mesothelin, demonstrated that mannose residues are present; excluding a role for metalloproteinases but not phospholipases [307].

We found that GDE3, unlike GDE2, hydrolyses the GPI anchor of the urokinase receptor (uPAR) in triple-negative breast cancer (TNBC). uPAR mediates degradation of the extracellular matrix through protease recruitment and enhances cell adhesion, migration and signaling through vitronectin binding and interaction with integrins. Expression is elevated in many human cancers and contributes to the aggressiveness of triple-negative breast cancer [120-122, 308]. As such, soluble uPAR derived from the tumor, stroma or infiltrating immune cells is often used as a marker for disease severity although the molecular mechanism behind uPAR shedding is unknown [120, 121, 128, 130-132, 309]. If GDE3 activity is fully responsible for the increase in total circulating GPI-anchor levels, observed in several cancer types, is questionable since we have found that GDE3 cleaves only a small uPAR fraction from the membrane. In addition, GDE3 expression was found to correlate with an increased survival probability in triple-negative breast cancer patients which contradicts soluble uPAR being a marker for disease severity. To assess the contribution of GDE3 in the total circulating uPAR levels, more research, focusing on the tissue origin (cancer cells or stroma) and type of circulating uPAR (cleaved or secreted), is needed.
Our results suggest that GDE3 cleaves uPAR at specific sites to elicit its effect. In accordance with this hypothesis, we find that GDE3 co-localizes with a known interactor of uPAR, integrin β3 (unpublished data) [123]. Although direct integrin activation by uPAR is proposed, Ferraris and colleagues showed that uPAR binding to Vitronectin activates integrin signaling in a non-canonical ligand-independent fashion [124]. The proposed model whereby uPAR, upon binding to vitronectin, creates membrane tension and in turn activates integrin signaling, fits with the previously described model of GPI-anchored proteins affecting cortical actin and membrane dynamics. Importantly, in both models, integrin activation is essential for uPAR induced morphological changes. Hence, cleaving uPAR in the proximity of integrins will disrupt the signaling axis and inhibit the uPAR induced morphology. If GDE3 releases uPAR from these specific sites remains to be explored.

The GDE-glypican signaling axis and their role in neuronal differentiation

GDE2 and GDE3 expression in the developing embryo is mutually exclusive, where GDE3 expression is restricted to glial precursors, GDE2 is mainly expressed in neurons. Previously, the retinoic acid-inducible gene, GDE2/GDPD5, was shown to be necessary and sufficient for motor-neuron differentiation during the embryonic development of the spinal cord [107, 108]. In contrast, the role of GDE3 during neuronal development remains largely unknown.

We found that GDE2 strongly correlates with a favorable clinical outcome for neuroblastoma, a childhood disease characterized by impaired neuronal differentiation [159-161]. Mechanistically GDE2 cleaves a small fraction of Glypican 6 in a cell-autonomous manner to suppress cell motility, oppose Rho-induced neurite retraction and alter gene transcription. We show that forced GDE2 overexpression pushes cells into differentiation and concomitant knockdown reversed the phenotype. Besides glypican 6 (GPC6), GDE2 also releases its family member glypican 3 (GPC3). However, unlike GPC6, does GPC3 knockdown not result in increased differentiation nor do expression levels correlate with a clinical outcome. Therefore, these findings raise the question of what determines GDE2 substrate recognition and how GPC6 specific cleavage leads to increased differentiation?

To date, six glypican family members have been identified which have a conserved overall structure but share only 25% sequence similarity [188]. Based on the primary structure, glypican 3 and 5 form one subgroup while glypican 1,2 and 6 constitute the other. From this, it remains unclear why GDE2 specifically cleaves GPC3 and 6 [188]. If heparin sulfates or the tertiary structures are involved in GDE2 substrate recognition warrants further investigation.
Although indirect, glypicans play an essential role in cellular signaling which is demonstrated by multiple genetic studies. As for GPC3, in mice, knockdown leads to overgrowth and limb defects reminiscent of phenotypes observed in Wnt or Bmp signaling defects [310-312]. Consistent with this, in humans, GPC3 and GPC4 function is conserved and mutations are associated with the Simpson-Golabi-Behmel syndrome (SGBS), characterized by postnatal overgrowth and morphological abnormalities [313-315]. Intriguingly, coherent to our observation that GPC6 inhibits differentiation in neuroblastoma, is GPC3 widely used as a marker for hepatocellular differentiation, in which high GPC3 staining reflects poorly differentiated hepatocellular carcinomas [316]. Whether GPC3 expression by itself is sufficient to keep cells in an undifferentiated state remains an open question.

Glypicans elicit their effect by modulating the kinetics of receptor activation in various ways. For example, they can stabilize ligand-receptor interactions to activate receptor signaling. Alternatively, through direct ligand binding, glypicans can concentrate the ligand at the target site to prolong receptor activation or create a gradient which is essential for cell polarity during drosophila development [317-320]. Interestingly more recent evidence suggests a possible role for glypicans as direct ligands for type II receptor protein tyrosine phosphatases (RPTPs) [199, 200]. Through their heparin sulfate proteoglycans, glypicans directly bind in cis to the ectodomains of RPTPs resulting in receptor clustering and consequent altered transmembrane signaling. Loss of GPC attenuates these clusters which leads to a change in localized phosphorylated tyrosine levels to stimulate neurite outgrowth. In accordance, we show that GDE2 localizes to the neurite tips where localized depletion of GPC6 could lead to decreased phosphor-tyrosine signaling and increased neurite formation.

Glypican 6 is arguably the least studied glypican and very little is known about the function. Though it was recently published that mutations in this gene lead to Autosomal-recessive omodysplasia (OMOD1) caused by defective hedgehog signaling [321]. Since GDE2 dependent cleavage of GPC6 drives neuroblastoma differentiation in cis, a role for Wnt signaling seems unlikely. If neuroblastoma differentiation, through GDE2 dependent release of GPC6, is indeed dependent on hedgehog or RPTP signaling is a subject for future studies. In conclusion, our findings uncover a role for GDEs in general and GDE2 specific in regulating GPI-APs at the membrane during neuronal differentiation. Further evidence for a role of GPI-specific phospholipases during neuronal differentiation is described in Addendum Chapter 3.
A non-neuronal role for GDE2

In Chapter 4 we identified the zebrafish orthologues (Gdpd5a and Gdpd5b) of human GDE2. Phylogenetic analysis revealed that Gdpd5a is closely related to human GDE2 while Gdpd5b is characterized by a relatively short, truncated C-terminal tail. Phenotypic analysis of zebrafish depleted of Gdpd5a by specific morpholino's (MOs) revealed anomalies similar to that observed in knockout mice and chicken, including defects in motility [110]. Overexpression of human GDE2, but not the catalytically dead GDE2, also gave rise to a curved body axis and motility defects, hinting to a tight balance and functional conservation for GDE2 transcription during development. Besides motility defects we also observed malformations in the heart and a dramatic reduction in pancreatic markers trypsin and insulin in the pancreas. Strikingly, other endocrine markers, such as pdx1, were not affected, which suggests a specific role for GDE2 in terminal differentiation of insulin-producing beta-cells. Also, in the exocrine pancreas, trypsin was strongly reduced while ptf1a was unaffected. In humans, GDE2 is also expressed in the pancreas which suggests that the function of GDE2 is conserved between the two species [252].

Upon retinoic acid induced transcription, GDE2 induces motor neuron differentiation through the release of GPI-anchored RECK from pre-motor neurons and concomitant inhibition of Notch signaling in neighboring cells [110]. It is of note that perturbations in the GDE2 effector, Notch, in zebrafish leads to inhibition of acinar cell differentiation but not initial commitment to the exocrine lineage [246]. In agreement with this, the GDE2 transcriptional activator retinoic acid, promotes endocrine at the expense of exocrine differentiation in the dorsal pancreas and correlates with specific inhibition of Notch signaling. Also, RA enhances exocrine marker gene expression in the ventral pancreas [322, 323]. Together these data suggest a possible role for the RA-GDE2-Notch signaling axis in the developing pancreas. Another possible mechanism, described in Chapter 3 includes the release of GPC6 by GDE2. Unfortunately, little is known about the function of GPC6 in zebrafish. However, depletion of its closest family member GPC4 in zebrafish, results in strongly reduced cardiomyocyte proliferation [255]. Also, consistent with our observations, in rat, glypicans are expressed during pancreas development and inhibition of proteoglycan sulphation using chlorate, results in a dramatic increase in beta cells and consequent insulin staining [324]. If the observed malformations upon Gdpd5a knockdown or human GDE2 overexpression are glypican- or RECK-specific is a subject for future studies.
Regulation and GDE substrate specificity

Throughout our studies we were interested in the substrate specificity of GDE2 versus GDE3. While both proteins are able to cleave GPI-anchored proteins, only GDE2 cleaves a specific subset of GPI anchors, revealing a second order substrate recognition beyond the GPI-anchor structure itself. Localization studies, described in Chapter 5, revealed that GDE2 is present in vesicles as well as on the membrane while GDE3 is mainly located on the membrane. This raises the question of whether GDE2 activity and membrane localization could be regulated through vesicular trafficking and recycling. In fact, previous studies proposed that activation of GDE2 is regulated in a redox dependent manner that involves vesicular trafficking. To this end, intracellular Peroxiredoxin 1 interacts with GDE2 to reduce an intramolecular disulfide bond that bridges the N- and C-terminus, leading to GDE2 activation and concomitant neuronal differentiation [118]. However, when we mutated the respective cysteine residues we did not observe any increase in activity nor did we observe a change in localization which raises the question if this is a critical step in the regulation of GDE2 (Chapter 3).

We hypothesized that although GDE2 is differentially regulated, its substrate recognition does not depend on the localization to specific plasma membrane domains. To verify this, we swapped the enzymatic domain of GDE2 with that of GDE3, leaving the GDE localization intact and analyzed GPI-AP release. Indeed, swapping the domain changes GDE2 specificity but not activity.

Because GDE2 is a GPI-specific phospholipase, its substrate is the GPI anchor and specificity must be determined by modifications within the GPI anchor itself (the potential modifications are discussed in Chapter 1) or by intrinsic properties of the attached protein. To test this, we fused the GPI anchor of GPC6 to uPAR and analyzed GDE2 and GDE3 activity. While GDE3 was able to cleave all constructs, GDE2 was unable to cleave uPAR. Together, these results suggest that besides GPI recognition, protein-protein interactions may determine substrate recognition by GDE family members.

Concluding remarks and perspectives

Although cleaved GPI-anchored proteins are readily detected in biological fluids, their origin and involved phospholipases have long been elusive. The studies presented in this thesis describe a novel role for the GDE family in selectively cleaving GPI-anchored proteins. Additionally, we show that GPI-anchor cleavage plays an important role in multiple (patho) physiological settings which opens up a new field of research. Thus far we have only focused on individual GDE family members and their role in shedding one specific GPI-AP, however,
the total subset of GPI-APs cleaved by GDE2 and GDE3 is unknown. We have shown that GDE's only cleave a small fraction from the membrane but with profound effects. Because GDE's cleave only a small fraction of the GPI-AP, efforts to identify substrates through mass spec analysis of the total membrane-bound GPI-anchored protein pool were until now unsuccessful. A new approach that detects the released protein, using a bacterial toxin (alpha toxin from *Clostridium septicum*) that specifically recognizes the sugar moieties of the GPI anchor, has proven to be more promising (data not shown). This, together with structural studies should give a better insight into how GDE's recognize and cleave their substrates. Another caveat in our current understanding of GDE's is how they are regulated. It seems unlikely that GDE's are continuously active to "shave" the membrane. In accordance to this, our localizations studies reveal specific intra-cellular localization, of GDE2 and GDE3, suggesting differential localized activation. A possible clue was observed in differentiating (polarized) neuronal cells where GDE2 preferentially localizes to the neurite tips, where GPI-anchored proteins may also reside [325]. While many questions remain to be addressed in the emerging GDE field of research, the new findings emphasize GPI-anchor hydrolysis by GDE's as a cell-intrinsic signaling mechanism to affect cell behavior and, in a broader context, help to clarify the biological significance of the once mysterious GPI anchors [326].